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Journal

JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, 139(1)

ISSN

0091-6749

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Publication Date

2017

DOI

10.1016/j.jaci.2016.02.035

Peer reviewed

Claudin-18 deficiency is associated with airway epithelial barrier dysfunction and asthma

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Funded by NIH/NHLBI R21HL111707 (JAF)

1 **ABSTRACT**

2 **Background:** Epithelial barrier dysfunction and increased permeability may contribute to antigen
3 sensitization and disease progression in asthma. Claudin-18.1 is the only known lung-specific tight junction
4 protein, but its contribution to airway barrier function or asthma is unclear.

5 **Objectives:** To test the hypotheses that claudin-18 is a determinant of airway epithelial barrier function that
6 is down regulated by IL-13, and that claudin-18 deficiency results in increased aeroantigen sensitization and
7 airway hyperresponsiveness.

8 **Methods:** Claudin-18.1 mRNA levels were measured in airway epithelial brushings from healthy controls
9 and asthmatics. In the asthmatics, claudin-18 levels were compared with a three-gene-mean marker of TH2
10 inflammation. Airway epithelial permeability changes due to claudin-18 deficiency were measured in 16HBE
11 cells and claudin-18 null mice. The effect of IL-13 on claudin expression was determined in primary human
12 airway epithelial cells and in mice. Airway hyperresponsiveness and serum IgE levels were compared in
13 claudin-18 null and wild type mice following aspergillus sensitization.

14 **Results:** Epithelial brushings from asthmatic subjects (n=67) had significantly lower claudin-18 mRNA
15 levels than healthy controls (n=42). Claudin-18 levels were lowest among TH2-high asthmatics. Loss of
16 claudin-18 was sufficient to impair epithelial barrier function in 16HBE cells and in mouse airways. IL-13
17 decreased claudin-18 expression in primary human cells and in mice. Claudin-18 null mice had significantly
18 higher serum IgE levels and increased airway responsiveness following intranasal aspergillus sensitization.

19 **Conclusions:** These data support the hypothesis that claudin-18 is an essential contributor to the airway
20 epithelial barrier to aeroantigens. Furthermore, TH2 inflammation suppresses claudin-18 expression,
21 potentially promoting sensitization and airway hyperresponsiveness.

22

23

24 **Key Messages**

25 • Claudin-18 is a key barrier-forming protein in the airway epithelium that is expressed at lower levels
26 in asthmatic subjects compared with healthy controls. TH2 inflammation markers are inversely associated
27 with claudin-18 levels in asthmatics.

28 • IL-13-mediated loss of claudin-18 may contribute to increased epithelial permeability, increased
29 sensitization to aeroantigens and airway hyperresponsiveness.

30

31 **Capsule Summary**

32 Epithelial barrier dysfunction is thought play a central role in asthma severity or progression. Decreased
33 expression of lung-specific claudin-18, due to TH2 inflammation, may promote sensitization to aeroantigens
34 and contribute to airway hyperresponsiveness.

35

36 **Key Words:** Asthma, Epithelium, Epithelial Barrier Function, Tight Junction, Antigen Sensitization, Airway
37 Hyperresponsiveness

38

39 **Abbreviations**

40 Asp: Aspergillus antigen

41 TER: Transepithelial electrical resistance

42 Papp: Apparent permeability

43 ZO-1: zona occludens-1

44

45 INTRODUCTION

46 Genetic and environmental factors influence asthma development, progression, and severity. Moreover,
47 recent work has begun to parse the clinical syndrome of asthma into distinct endotypes that may vary in
48 pathogenesis, progression and response to therapy (1). Amid this complexity, airway epithelial barrier
49 impairment is a common feature of asthma that has been postulated to contribute to immune and
50 parenchymal cell activation, antigen sensitization, and airway hyperresponsiveness (2, 3). In severe
51 asthma, epithelial cell loss has been reported, but prior work has also demonstrated that more subtle
52 changes in epithelial cell junctions may account for impaired barrier function in mild-moderate asthma (4).
53 For example, infections, toxins and environmental proteins, such as Dpr1, modify cell junctions to impair
54 barrier function (5-7). Moreover, recent work also supports the hypothesis that differences in tight junction
55 protein expression or trafficking account for differences in barrier function in the asthmatic epithelium. For
56 example, biopsy samples from asthmatic subjects exhibit tight junction disruption with reduced expression
57 of occludin and ZO1 — key structural components of tight junctions (8). In parallel, cultured epithelial
58 monolayers derived from asthmatic airways had increased macromolecule permeability compared with
59 monolayers derived from healthy subjects (8). Because the airway epithelium constitutes a barrier to
60 aeroantigens, these findings raise the possibility that increased epithelial permeability could contribute to
61 allergic inflammation by permitting greater exposure of the subepithelial compartment to inhaled allergens.
62 Alternatively, loss of epithelial compartmentalization and polarity could impact cell signaling through
63 dysregulation of normally segregated receptors and ligands. The mechanisms of airway epithelial tight
64 junction dysfunction in asthma, and the contribution of this abnormality to allergic sensitization and airway
65 hyperresponsiveness remain incompletely understood.

66 It is noteworthy that previous studies have established that TH2-mediated changes in tight junction claudin
67 expression contribute to impaired epithelial barrier function in the gut and other organs (9-11). It is less clear
68 whether this mechanism contributes to airway epithelial barrier abnormalities in asthma; however, one
69 previous report found that IL-4 and IL-13 impaired epithelial barrier function in 16HBE cells (12). TH2-high
70 asthma is characterized by higher expression levels of IL-13-dependent genes, greater airway

71 hyperresponsiveness, and higher IgE levels compared with TH2-low asthmatics (13, 14). It remains unclear
72 whether tight junction composition and epithelial barrier function differ among asthmatics with TH2-high and
73 TH2-low asthma.

74 Claudins are essential to tight junction formation and are a primary determinant of paracellular permeability
75 through intact tight junctions. Differential claudin expression accounts for the differences in epithelial
76 permeability in diverse epithelia, and mutations in claudin genes and changes in claudin expression result in
77 clinical disease (15, 16). The claudin-18 gene encodes two variants that differ in the first exon. Claudin-18.1
78 expression requires the transcription factor NKX2-1 and is exclusive to lung epithelia (17). At present,
79 claudin-18.1 is the only known lung-specific tight junction gene product. The expression of specific claudin
80 family members may vary in different airway epithelial cell types, but claudins-1, -4, and -7 are expressed at
81 high levels in airway epithelium (18, 19). Although claudin-18 is also normally expressed in airway epithelial
82 cells, its contribution to the permeability barrier in the airways has not been fully defined.

83 This study was undertaken to determine the contribution of claudin-18 to airway epithelial barrier function
84 and whether claudin-18 expression is regulated by TH2 inflammation. In addition, the consequences of
85 claudin-18 deficiency to aeroantigen sensitization and airway hyperresponsiveness were examined. The
86 presented data substantiate the hypothesis that claudin-18 is a central barrier-forming component of tight
87 junctions and show that IL-13 downregulates claudin-18. These data also suggest that the loss of claudin-
88 18 is associated with increased sensitization to aeroantigens and airway responsiveness.

89

90 **METHODS**

91 Airway mRNA Expression Studies

92 Aliquots of RNA extracted from airway epithelial brushings and stored in the Airway Tissue Bank (ATB) at
93 the University of California, San Francisco were analyzed by qPCR as previously described (20). The
94 UCSF Committee on Human Research approved the policies and procedures of the UCSF ATB and use of

95 samples for this study. These epithelial brushings had been collected during research bronchoscopy from
96 67 nonsmoking subjects with asthma and 42 healthy nonsmoking control subjects (Table 1). All subjects
97 signed an ATB informed consent form approved by the UCSF Committee on Human Research. Asthmatic
98 subjects had a prior physician's diagnosis of asthma, a PC20 methacholine value of less than 8 mg/mL, and
99 were using only inhaled β -agonist medications for therapy. RNA was reverse transcribed with random
100 hexamer primers and then amplified in a multiplex reaction with custom primers (Table S1). The amplified
101 cDNA was then used for qPCR with custom primers and Taqman-based probes run in separate batches
102 (13, 21). Normalization was done using the geometric mean of the value of three housekeeping genes as
103 previously described (21, 22). Log₂ transformed, normalized, relative expression is reported. The three-
104 gene-mean marker of Th₂ inflammation was determined as described previously (13, 14). This measure is
105 based on the geometric mean of mRNA expression levels of CLCA1, SERPINB2, and POSTN. Claudin-18
106 mRNA expression levels were compared with serum IgE levels and blood eosinophil counts obtained from
107 asthmatic subjects at the time of bronchoscopy .

108

109 Primary Cell Culture and Immunostaining

110 Cadaveric airway tissues from lungs rejected for transplantation were obtained in accordance with UCSF
111 Committee on Human Research approval from the Northern California Organ Donor Network. Normal
112 airway surface epithelial cells were harvested from 8 individual donors, expanded and cultured at an air-
113 liquid interface(ALI) as described previously (23, 24). Treatment with IL-13 (10 ng/ml) was begun at two
114 weeks, once ALI cultures had established confluence, and continued for 7 days. Recombinant human IL-13
115 (R&D Systems) was reconstituted in sterile PBS containing 0.1% bovine serum albumin. For primary human
116 airway epithelial cells, qPCR was done without preamplification. Normalization was done as above using
117 EEF1A1 and PPIA. Data are reported as relative mRNA expression normalized to the housekeeping genes.
118 Claudin-18 protein levels were compared in cell lysates using immunoblot densitometry normalized to
119 tubulin or GAPDH. Blots were incubated with claudin-18 and tubulin primary antibodies in series and

120 images developed such that both bands could be visualized on the same blot. In addition, separate, equally
121 loaded blots were probed for either claudin-18 or GAPDH. Data normalized to tubulin or GAPDH were
122 similar and the GAPDH data are shown. For immunostaining, tissue was fixed in 4% paraformaldehyde and
123 embedded in paraffin. Tissue sections were stained for beta catenin (G10, Santa Cruz Biotechnology) and
124 claudin-18 (ZMD.385, Life Technologies) and counterstained with DAPI.

125

126 16HBE Cell Culture

127 16HBE cells (a gift from Dr. Dieter Gruenert, UCSF) were cultured in MEM Eagle's with Earle's BSS
128 Medium supplemented with 10% BSA, 1% penicillin/streptomycin and 1% glutamine. A coating medium
129 comprised of LHC basal media, 0.1% type 1 collagen, 0.1% BSA, 0.1% fibronectin was used to coat the
130 culture plates. Cells were cultured until they reached 80% confluence and 250,000 cells/cm² were then
131 passaged on to 1.13-cm² Transwell polycarbonate inserts (3407, Corning Costar) treated with coating
132 medium. Claudin-18 and tubulin protein expression levels were measured using Western blotting
133 densitometry data were analyzed using ImageJ (NIH, Bethesda, MD). Data are reported as claudin-
134 18/tubulin. Transepithelial electrical resistance was measured using a voltohm meter (World Precision
135 Instruments). Permeability to the 500 Dalton fluorescent tracer pyranine was measured by adding 10ug/ml
136 pyranine to the apical chamber of transwells and measuring fluorescence recovery in the basolateral
137 chamber (25). Data are reported as apparent permeability.

138

139 shRNA Studies

140 Claudin 18 shRNA sequences (TRCN0000116737 to TRCN000011673741) were cloned into the 3rd
141 generation lentiviral vector pLKO.1-puro (Sigma Mission shRNA library) and transfer plasmids were
142 cotransfected with packaging plasmids and VSV-G expressing envelope plasmid into Human 293 cells at
143 low passage and 30% confluence. When the cells became confluent (36-48h), the culture medium was

144 collected and filtered. Supernatants were aliquoted and kept at -80C. After confirmation of post-transduction
145 knockdown by immunoblot, transepithelial electrical resistance (TER) of stable monolayers and paracellular
146 permeability to the 0.5 kDa fluorescent tracer pyranine were measured as previously described (25).

147

148 Mouse Studies

149 Animal studies were done in accordance with local Institutional Animal Research Committee (IACUC)
150 approval. Constitutive Cldn18^{-/-} mice on the C57/b6 background were generated by the trans-NIH Knock-
151 Out Mouse Project. The knock out strategy targeted a 17 kB DNA genome segment encompassing the 5
152 exons of claudin-18.1. Isoflurane-anesthetized mice were exposed to aspergillus 100ug antigen (Hollister) in
153 40 ul saline or saline alone intranasally three times per week for 3 weeks as previously described (26). One
154 day after the final aspergillus administration, mice were anesthetized with ketamine/acepromazine (90/2
155 mg/kg) and received pancuronium (1 mg/kg). Airway resistance was measured with a FlexiVent system
156 (Scireq) before and after increasing doses of acetylcholine (i.v.). Total serum IgE was measured as
157 previously described (26). Separate claudin-18 null and wild type mice were sensitized intra-peritoneally
158 with 20 mg of Aspergillus mixed with 20 mg of aluminum potassium sulfate (alum) on day 0 and 14. Control
159 animals received an equal volume of alum alone. Mice were euthanized for serum harvesting 3 days after
160 the second sensitization. Additional mice were sensitized on days 0, 7, and 14 by intraperitoneal injection of
161 50 ug ovalbumin (OVA, Sigma Aldrich) emulsified in 1 mg of alum. Control animals received an equal
162 volume of alum alone. Mice were euthanized for serum harvesting 7 days after the third sensitization. OVA-
163 specific IgE levels were measured by ELISA using microplates coated with OVA. Diluted serum samples
164 were added to each well, and the bound IgE was detected with biotinylated anti-mouse IgE (R35-118;
165 Pharmingen).

166 To further explore the effect of IL-13 on claudin-18 expression in vivo, previously described IL-13 over-
167 expressing transgenic mice and wild type C57/b littermate controls were compared (27, 28). Lung tissue

168 was collected and mRNA and protein extracted. Claudin-18 mRNA and protein abundance was determined
169 as described above.

170 Tracheal epithelial permeability to macromolecules was measured as previously described (29) with slight
171 modification. Briefly, tracheas from claudin-18 null and wild type littermates were cannulated at both ends
172 with PE50 tubing and instilled with 50 ul of Krebs buffer containing 1mg/ml of the 0.5 kD fluorophore
173 pyranine. Temperature was maintained at 37°C for 30 minutes. Tracheas were then gently flushed with 300
174 ul of tracer-free Krebs buffer and homogenized in 250 ul of fresh buffer. Prior to homogenization, tracheal
175 segment width and length, spanning the distance between the sutures securing the PE50 tubing, were
176 recorded for determination of tracheal epithelial surface area. Homogenates were centrifuged for 10 min at
177 12,000 g and fluorescence was measured in 100 ul aliquots of supernatant. Tracheal epithelial permeability
178 is reported as apparent permeability (P_{app}) or $(dQ/dT)/CA$, where dQ/dT is the permeation rate of tracer as
179 a mass per time, C is the initial concentration of tracer in the buffer and A is the tracheal epithelial surface
180 area.

181

182 Histology

183 Lungs were inflated with 10% buffered formalin to a pressure of 25 cm H₂O. Sections (5 μm) of the entire
184 lung were stained with hematoxylin and eosin, periodic acid-Schiff (PAS), or with Sirius red. PAS staining
185 was scored in a semi-quantitative fashion as previously reported (30). Airway fibrosis was quantified using
186 the area/perimeter method as previously reported (31).

187

188 Statistics

189 Comparisons between two groups were done by t-test for normally distributed data or by the Wilcoxon rank
190 sum test. Claudin-18 mRNA expression in airway brush samples was compared using ANOVA with posttest
191 for linear trend and post hoc Bonferroni correction for multiple comparisons. For airway resistance data,

192 ANOVA and pair-wise, post hoc Bonferroni correction was done. For primary cell culture studies,
193 Friedman's test with Dunn's correction for multiple comparisons was used. P values < 0.05 were considered
194 statistically significant. Statistics were calculated using Prism v6.04 (GraphPad Software). Data are shown
195 as mean +/- SEM unless otherwise indicated.

196

197 **RESULTS**

198 *Claudin-18 expression is decreased in asthma and is inversely associated with TH2 inflammation*

199 Claudin-18 mRNA levels were lower in epithelial brush samples from subjects with asthma (n=67) as
200 compared to healthy controls (n=42) (Figure 1A). Claudin-18 levels were lower in asthmatics with low three
201 gene mean values compared with healthy controls and were lowest in the asthmatic subjects with higher
202 three-gene-mean values (P<0.0001 by ANOVA with a significant linear trend). TH2-high asthmatics (n=44)
203 had the lowest claudin-18 mRNA levels (P<0.05 compared with healthy subjects following post-hoc
204 Bonferroni test for multiple comparisons). Consistent with previous reports (13), the majority of asthmatics
205 were TH2-high (44/67) as defined by a three-gene-mean of greater than 0.1. Although protein samples were
206 not available from the epithelial brush samples used in this study, the presence of claudin-18 protein was
207 examined in airway epithelium from non-asthmatic tracheal tissue (Figure 1B). Claudin-18 immunolocalized
208 to the epithelium as defined by the presence of beta catenin. Among asthmatic subjects, there was a
209 modest inverse correlation between serum IgE levels and claudin-18 levels in the airway brush samples (rs
210 = -0.31)(Figure 1C). There was also an inverse correlation between claudin-18 levels and blood eosinophil
211 counts among the asthmatic subjects (rs = -0.42)(Figure 1D).

212 *IL-13 decreases claudin-18 mRNA and protein levels in primary human airway epithelial cells*

213 In primary human airway epithelial cells IL-13 induced a significant decrease in claudin-18 protein
214 expression compared with vehicle-treated controls (Figure 2A). IL-13 significantly decreased claudin-18

215 mRNA levels, but did not significantly change mRNA levels of claudin-1, -4 or -7 (n = 8 separate
216 donors)(Figure 2B).

217

218 *Loss of claudin-18 impairs epithelial barrier function*

219 In 16HBE cells, claudin-18-targeted shRNA decreased claudin-18 protein levels by approximately 50%
220 (Figure 3A), but did not significantly affect protein levels of claudin-1, -4 or -7. Claudin-1 protein expression
221 in cldn18-targeted shRNA-treated cells was $110 \pm 20\%$ of control shRNA-treated cells. Claudin-4 protein
222 expression was $91 \pm 15\%$ and claudin-7 protein expression was $114 \pm 10\%$ of control shRNA-treated cells
223 based on densitometry normalized to tubulin (n=6 biologic replicates).

224 Knock down of claudin-18 resulted in a significant decrease in transepithelial electrical resistance and a
225 significant increase in epithelial permeability to the 0.5 kD tracer pyranine in 16HBE cells ($P < 0.05$)(Figure
226 3B and C).

227 Tracheal epithelial permeability to pyranine was assessed in wild type and claudin-18 null mice (n=6 in each
228 group). This method yielded permeability values comparable to previous studies of airway permeability in
229 wild type mice ($P_{app} = 3.3 \pm 0.3 \times 10^{-7}$ cm/sec). Consistent with the in vitro claudin-18 knock down studies,
230 tracheal epithelial permeability was more than 2-fold higher in claudin-18 null mice ($P < 0.05$) (Figure 4D).

231

232 *Claudin-18 deficiency increased sensitization to aeroantigens and airway responsiveness in mice*

233 To investigate the association between claudin-18, epithelial barrier function and asthma, claudin-18 knock
234 out mice were exposed to intranasal aspergillus antigen. Following the final intranasal exposure, serum IgE
235 levels and airway resistance changes in response to intravenous acetylcholine were measured. Compared
236 with wild type mice, claudin-18 null mice showed increased airway responsiveness to acetylcholine

237 following intranasal aspergillus sensitization (Figure 4A)($P < 0.01$). Control, saline treated, claudin-18 knock
238 out mice were not different from saline-treated wild type mice ($n=6-8$ in each group).

239 Serum IgE levels in intranasally sensitized *cldn18* null mice were significantly higher than aspergillus-
240 treated wild type mice and saline-treated control mice (Figure 4B). To determine if the airway route of
241 administration accounted for the difference in sensitization to aspergillus, additional mice were sensitized to
242 aspergillus antigen using the intraperitoneal route. Serum IgE levels were not different in knock out and wild
243 type mice given intraperitoneal aspergillus antigen (Figure 4C). To further evaluate for baseline differences
244 in antigen sensitization, additional mice were sensitized to ovalbumin via the intraperitoneal route and ova-
245 specific IgE was measured in serum. Ova-specific IgE levels were not different between knock out and wild
246 type mice (Figure 4C).

247 On lung histology, there was no difference in airway mucin between genotypes at baseline as quantified by
248 PAS staining. Intranasal aspergillus sensitization increased PAS staining to a similar degree in both
249 genotypes (Figure 4D). Similarly, aspergillus sensitization increased airway collagen to a comparable
250 degree in both genotypes. Although airway collagen was slightly higher in knock out mice after aspergillus,
251 the difference did not reach statistical significance ($n=6-8$ in each group)(Figure 4E).

252

253 *IL-13 overexpression in vivo decreases claudin-18 mRNA and protein expression*

254 To test whether IL-13 decreases lung claudin-18 expression in vivo, previously described transgenic IL-13
255 overexpressing mice were compared with wild type littermates. Claudin-18 protein expression, normalized
256 to tubulin, was significantly decreased in IL-13 transgenic mice compared with wild type mice (Figure 5A).
257 Using qPCR, claudin-18 mRNA levels, normalized to beta actin, were significantly decreased to a similar
258 degree (Figure 5B). Claudin-4 mRNA expression was significantly increased in IL-13 overexpressing
259 transgenic mice and claudin-1 and claudin-7 mRNA levels were not different between the genotypes ($n=6$ in
260 each group) (Figure 5B).

261

262 **DISCUSSION**

263 The overall objective of this study was to establish the role of claudin-18 in airway epithelial barrier function
264 and determine whether claudin-18 is regulated by mediators of TH2 inflammation. Based on the hypothesis
265 that claudin-18 is a requirement for the epithelial permeability barrier, the contribution of claudin-18 to
266 aeroantigen sensitization and airway responsiveness was investigated with the prediction that serum IgE
267 and airway responsiveness would be increased in the setting of claudin-18 deficiency. The primary findings
268 of this study are that claudin-18 levels are decreased in asthmatic subjects in association with TH2
269 inflammation, and that IL-13 acts to decrease claudin-18 expression in primary human cells and in mice. In
270 addition, asthmatic subjects with low claudin-18 mRNA levels had higher serum IgE levels and blood
271 eosinophil counts. Claudin-18 deficient mice developed a greater increase in serum IgE following
272 intranasal, but not intraperitoneal aspergillus antigen sensitization. Claudin-18 deficient mice also developed
273 greater airway hyperresponsiveness compared with wild type mice following intranasal aspergillus
274 sensitization. Together these data support the hypothesis that claudin-18 is a requirement for airway
275 epithelial barrier function and suggest that increased epithelial permeability may contribute to greater
276 antigen sensitization and airway responsiveness in asthma. Because TH2 inflammation decreases claudin-
277 18 expression, the data in this study are consistent with the hypothesis that TH2 inflammation and epithelial
278 barrier dysfunction can participate in a feed forward loop in asthma that includes the down regulation of
279 claudin-18.

280 The contributors to disease development and severity in asthma are manifold; however, epithelial
281 barrier dysfunction may represent a common physiological feature of asthma that is downstream to diverse
282 genetic and environmental inputs. Compromised epithelial permeability function represents a partial loss of
283 compartmentalization and potentially epithelial polarization as well. These changes in epithelial function
284 may permit increased sensitization to aeroantigens and alter cell signaling pathways that are normally
285 regulated by segregated receptors and ligands. There are several examples of the causative role epithelial

286 barrier dysfunction can play in disease progression outside of the airway (32, 33) (10). TH2 inflammation
287 appears to be a potent modulator of epithelial permeability, not only in the gut, but also the skin, nasal
288 epithelium, and 16HBE cells (9, 11, 12). In the context of asthma, previous studies have reported decreased
289 expression levels of certain junctional proteins, including epithelial cadherin, occludin and ZO-1 (8, 34-36).
290 Prior work has also shown that innate immune mediators such as TNF-alpha can influence claudin
291 expression in airway epithelial cells (18). The data from the present study and others implicate a crucial role
292 for epithelial barrier dysfunction in asthma.

293 To date, relatively little is known about the regulation of claudins in asthma or the consequences of
294 any changes in claudin expression to disease. Although numerous proteins make contributions to the
295 epithelial permeability barrier, the claudin family of proteins has particular importance. Claudins form the
296 band-like meshwork within the apical junction complex that accomplishes the sealing function between cells
297 as well as epithelial polarization within the membrane. Claudin-18.1 is of interest because it is uniquely
298 expressed in lung epithelia. The recent development of claudin-18 null mice has begun to advance our
299 understanding of the function of this protein in the lung. For example, claudin-18 null mice exhibit alveolar
300 epithelial barrier defects. In the alveolar epithelium, loss of claudin-18 results in increased paracellular
301 permeability and altered tight junction structure between type 1 pneumocytes (37, 38). In cultured alveolar
302 epithelial cells the permeability-limiting function of claudin-18 is not replaced by increased expression levels
303 of other claudin family members (37). Interestingly, claudin-18 deficiency results in impaired alveolarization
304 postnatally; the phenotype of claudin-18 null mice shares some similarity with animal models of
305 bronchopulmonary dysplasia (37). Although claudin-18 is expressed in the airways, the contribution of
306 claudin-18 to airway barrier function has not been previously reported in detail. Ongoing development of
307 conditional claudin-18 knock out mice may help to separate the effects of claudin-18 deficiency on the
308 alveolar and airway epithelium, but the data presented in this study indicate that airway epithelial
309 permeability is higher in the absence of claudin-18.

310 A key finding of this study is that claudin-18 mRNA levels are reduced in the airways of asthmatic
311 subjects and that claudin-18 expression is inversely associated with TH2 inflammation (Figure 1A). These

312 data are consistent with the hypothesis that IL-13 suppresses claudin-18 expression in airway epithelial
313 cells. The observed decrease in claudin-18 mRNA levels could be the result of a direct effect of IL-13 on
314 claudin-18 mRNA expression or stability. Alternatively, this finding could be the result of a change in the
315 relative abundance of particular cell populations within the epithelium that differ in claudin-18 expression
316 levels. Claudin-18 protein levels were not examined in this study because the airway brush specimens used
317 were too small to allow for protein-level analysis. However, there was a consistent correlation between
318 claudin-18 mRNA and protein levels in primary airway epithelial cells and in 16HBE cells.

319 Atopic status is an important phenotypic feature of asthma that influences treatment decisions, but
320 the mechanisms underpinning the association between atopy and asthma pathogenesis or progression are
321 not entirely understood. Because environmental antigens drive TH2 inflammation in atopic individuals,
322 increased antigen exposure due to epithelial barrier dysfunction could potentially increase asthma severity
323 in these patients. The findings of this study raise the possibility of an inverse association between claudin-
324 18 levels and sensitization to inhaled environmental antigens. It should be acknowledged that the
325 magnitude of antigen exposure is not the only determinant of serum IgE or eosinophilia, and differences
326 among subjects with asthma could result from other factors, including TH2 status.

327 To more directly test the effect of IL-13 on claudin-18 expression, primary human airway epithelial
328 cells were cultured in the presence of IL-13. The effect of IL-13 on claudin-18 expression was also
329 examined in vivo in IL-13 overexpressing transgenic mice. Although mouse models of asthma should be
330 interpreted with caution, these mice are known to exhibit airway hyperresponsiveness and increased serum
331 IgE levels following antigen exposure (27, 28). In each of these experiments, IL-13 decreased claudin-18
332 levels. In contrast to the studies in primary human airway epithelial cells, claudin-4 mRNA levels were
333 significantly higher in IL-13 transgenic mice compared with wild type mice. These data suggest that IL-13
334 exposure results in additional changes in airway claudin expression in mice. Barrier function was not
335 assessed in this experiment, but prior work has demonstrated that IL-13 impairs airway epithelial barrier
336 function (12).

337 To better understand the specific contribution of claudin-18 to airway epithelial barrier function, the
338 human airway epithelial cell line 16HBE was used in loss-of-function studies. 16HBE cells form tight
339 junctions and have been used as a model of the airway epithelial barrier (39). In these cells, the loss of
340 claudin-18 is sufficient to impair epithelial barrier function, including increased permeability to
341 macromolecules. In additional experiments, airway epithelial permeability was examined in intact tracheas
342 from claudin-18 null and wild type mice in situ. Similarly, claudin-18 null mouse tracheas had significantly
343 higher permeability. The mechanism by which claudin-18.1 uniquely limits epithelial permeability is not yet
344 certain. In stomach epithelium, expression of claudin18.2 results in the formation of a distinct apical tight
345 junction strand (40); however, previous studies of airway epithelial tight junction ultrastructure have not
346 reported distinct apical strands analogous to those reported in stomach epithelium (41, 42). Claudin-18.1
347 (lung) and -18.2 (stomach) differ in sequence at the first of two extracellular domains. Although claudin-18.1
348 deficiency alters alveolar epithelial type 1 cell tight junction structure (37), it is not yet clear if claudin-18.1
349 deficiency results in ultrastructural changes to airway epithelial tight junctions. It remains possible that the
350 loss of claudin-18 results in larger changes in tight junction organization or composition that compromise the
351 permeability barrier in the airway. Although the structural relationship between claudin-18 and tight junction
352 function requires additional investigation, the data from the present study demonstrate that claudin-18 is a
353 requirement for normal airway epithelial permeability.

354 The contribution of claudin-18 deficiency to aeroantigen sensitization and airway
355 hyperresponsiveness was examined in claudin-18 null mice using the intratracheal aspergillus sensitization
356 model. Because claudin-18.1 expression is restricted to the lung epithelium, if the barrier defect resulting
357 from claudin-18 deficiency augments antigen sensitization, it would follow that sensitization to antigens
358 delivered via a route other than the airway would be similar in wild type and claudin-18 null mice. Therefore,
359 mice were also sensitized with intraperitoneal aspergillus and serum IgE levels were measured. In contrast
360 to the results of the intraairway sensitization experiments, wild type and claudin-18 null mice had similar
361 serum IgE levels following intraperitoneal aspergillus or ova sensitization (Figure 4C). These data indicate
362 that sensitization responses in claudin-18 null mice are not intrinsically different from wild type mice, but

363 sensitization to aeroantigens is greater in the absence of claudin-18. Although airway responsiveness and
364 serum IgE levels differed between wild type and knock out mice, mucous cell hyperplasia and airway
365 fibrosis did not significantly differ following airway aspergillus sensitization (Figure 4D and E). In sum, these
366 mouse studies show that claudin-18 deficiency results in increased airway permeability and increased
367 serum IgE levels following intraairway, but not intraperitoneal antigen exposure. Murine models of asthma
368 have important limitations. Mouse models do not necessarily recapitulate responses observed in human
369 subjects or tissues. Although the data from this study show a significant increase in airway permeability in
370 human cells with claudin-18 depletion, the sensitization responses observed in the mouse and human
371 studies may not result from identical mechanisms. A growing body of evidence has focused attention on
372 airway epithelial barrier dysfunction as a central feature of asthma. The potential contributions of increased
373 epithelial permeability and loss of epithelial polarity to asthma include heightened exposure to air space
374 antigens and altered cell signaling due to the loss of segregation of normally polarized receptors and
375 ligands. These abnormalities may serve to propagate allergic inflammation, increase parenchymal cell
376 activation and promote hyperresponsiveness. Previous studies have found that specific structural
377 components of cell-cell junctions exhibit decreased abundance or abnormal localization in the airways of
378 asthmatic subjects. Data from this study suggest that decreased claudin-18 expression mediated by IL-13 is
379 an additional junctional abnormality in some asthmatics, particularly those with TH2-high asthma. Claudin-
380 18 appears to play a non-redundant role in the epithelial permeability barrier, potentially through effects on
381 tight junction organization, or a more selective effect on paracellular permeability. A better understanding of
382 the regulation of claudin-18 in disease may provide new insights for targeted therapeutics.

383 **FIGURE LEGENDS**

384

385 **Figure 1.** Airway claudin-18 expression in asthmatics. **A)** Claudin18.1 levels in asthmatics and healthy
386 controls. Claudin-18.1 levels are lowest in TH2-high asthmatics, $P < 0.0001$ by ANOVA and significant
387 posttest for linear trend. **B)** Claudin-18 immunolocalized to the epithelium (beta catenin positive cells) in
388 tracheal tissue. **C)** Serum IgE and **D)** blood eosinophils are inversely correlated with airway claudin-18
389 levels in asthmatics.

390 **Figure 2.** IL-13 decreases claudin-18 expression in primary human airway epithelial cells. **A)** IL-13
391 decreased claudin-18 protein levels (representative blot shown, brackets indicate cells from the same
392 donor)($n=8$, $*P < 0.05$ by paired analysis). **B)** IL-13 decreased claudin-18.1 mRNA levels, but did not
393 significantly change claudin-1, -4 or -7 mRNA abundance ($n=8$, $*P < 0.05$).

394 **Figure 3.** Claudin-18 is required for the airway epithelial permeability barrier. **A)** Claudin-18-shRNA
395 decreased claudin-18 protein levels in 16HBE cells ($*P < 0.05$). Representative blot above with densitometry
396 ($n=6$) below. **B)** Claudin-18 knock down decreased transepithelial electrical resistance (TER) and **C)**
397 increased apparent permeability (P_{app}) ($*P < 0.05$). **D)** Tracheal epithelial permeability in claudin-18 null and
398 wild type mice ($n=6$ in each group, $*P < 0.01$).

399 **Figure 4.** Airway responsiveness and serum IgE levels after aspergillus (Asp) sensitization in mice. **A)**
400 Following intra-nasal aspergillus sensitization, airway resistance responses were greater in claudin-18 null
401 mice ($*P < 0.05$). **B)** Serum IgE following intra-nasal aspergillus ($*P < 0.05$) and **C)** intraperitoneal (ip)
402 aspergillus or ova. **D)** PAS staining and **E)** collagen deposition before and after intra-nasal aspergillus
403 sensitization ($*P < 0.05$ versus saline).

404 **Figure 5.** IL-13 decreases claudin-18 expression in mice. **A)** Mice hemizygous for a transgene that
405 overexpresses IL-13 (IL-13 TG) have lower claudin-18 protein ($*P < 0.05$) and **B)** mRNA levels than wild type
406 littermates.

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Table. Airway brush specimen subject data.

	Healthy	Asthma
Sample Size	42	67
Female	22	35
Age	34 (27 – 40)	33 (24 – 42)
BMI	25.7 (22.6 – 29.6)	26.9 (23.9 – 29.5)
FEV1 (percent)	102.5 (94.3 – 113.5)	85.4 (79.5 – 94.0)*
FVC (percent)	108.2 (99.7 – 115.0)	100.0 (92.4 – 109.0)*

Data are median and 25-75% quartile range (*P < 0.05 by Wilcoxon rank-sum test for each pair).

Figure 1

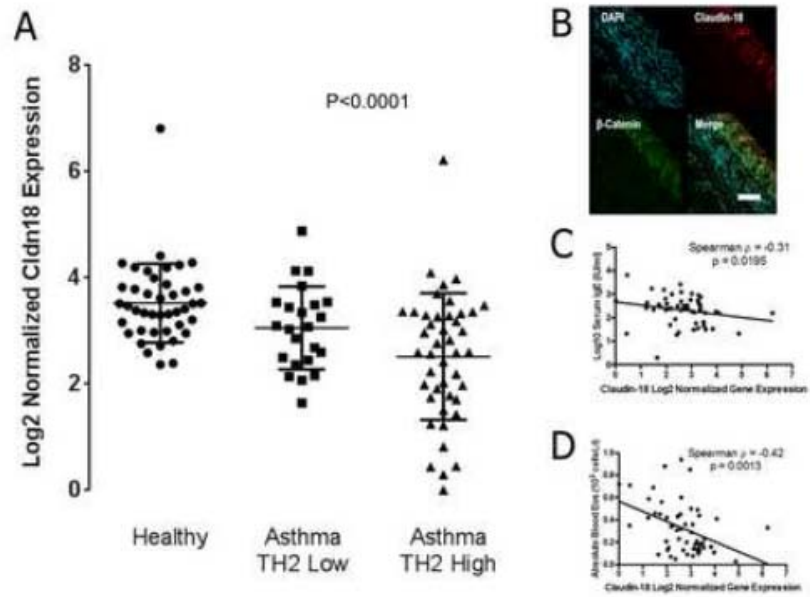


Figure 2

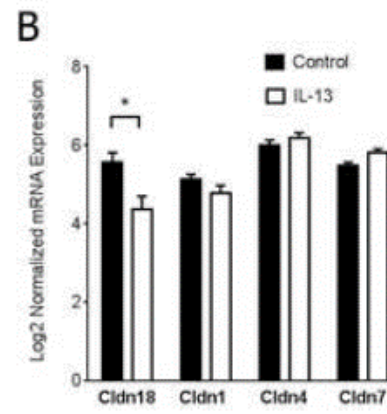
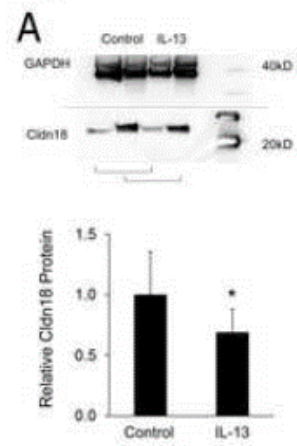


Figure 3

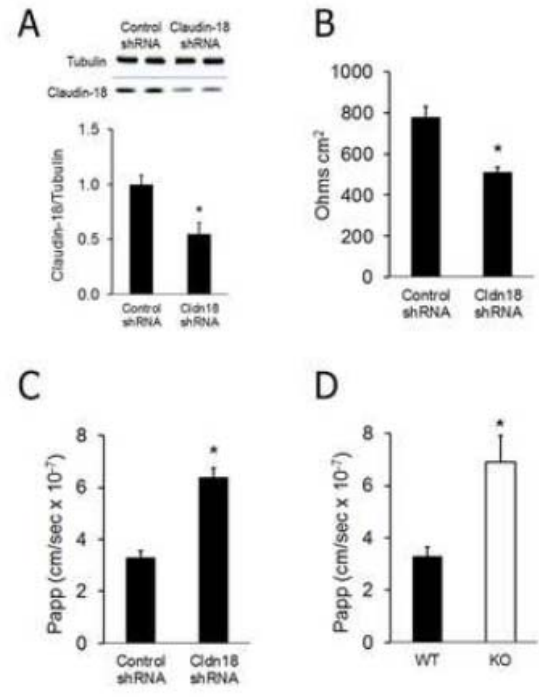


Figure 4

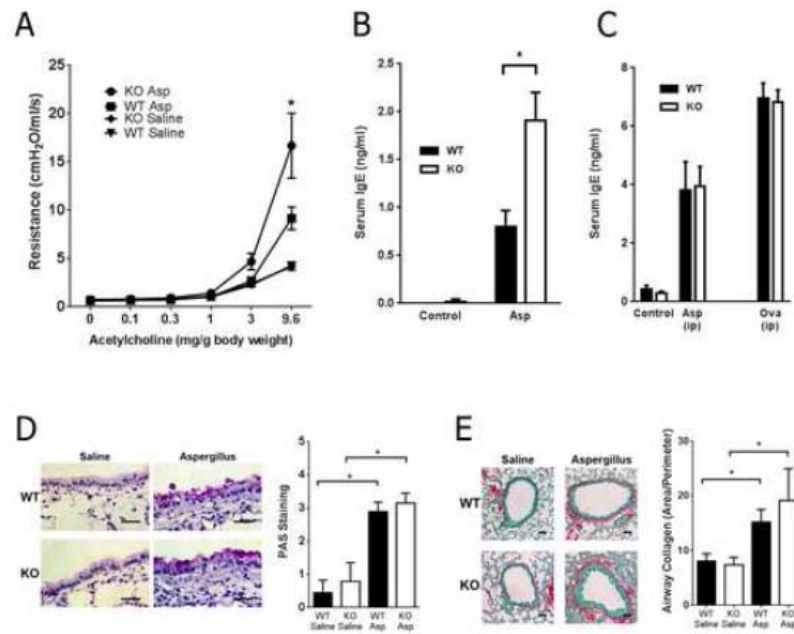


Figure 5

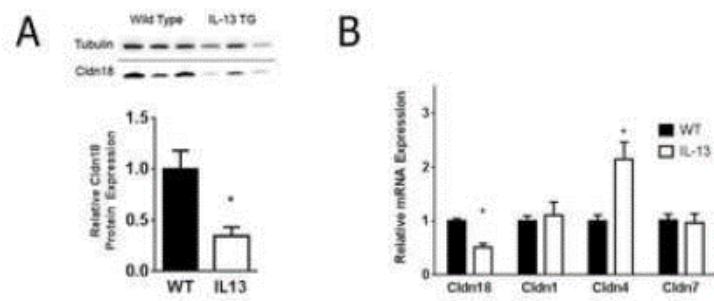


Table. PCR primers and probes.

	Gene	Probe	Sequence
<i>Claudin Family</i>	CLDN1	Outer Forward	CCACAGCATGGTATGGCAATAG
		Outer Reverse	TGGTGTGGGTAAGAGTTGTTT
		Inner Forward	CAGTCAATGCCAGGTACGAATTT
		Probe	TCAGGCTCTTCACTGGCTGGGC 5-FAM/3-BHQ
		Inner Reverse	AAGTAGGGCACCTCCCAGAAG
	CLDN4	Outer Forward	GAGATGGGTGCCTCGCTCTAC
		Outer Reverse	AAGAACAAGCAGAGAGGAACAGAGT
		Inner Forward	GGCTGCTTTGCTGCAACTG
		Probe	CCACCCGCACAGACAAGCCTT 5-FAM/3-BHQ
		Inner Reverse	CAGAGCGGGCAGCAGAATAC
	CLDN7	Outer Forward	TGATGAGCTGCAAATGTACGA
		Outer Reverse	CAGCGCTGCACTTCATG
		Inner Forward	GGCCAATCGAGCCCTAATG
		Probe	CACAAATCGGCCAGGAAGCCCA 5-FAM/3-BHQ
		Inner Reverse	TGCACTTCATGCCATCGT
CLDN18.1	Outer Forward	CCTATTTACCATCCTGGGACTT	
	Outer Reverse	GAGGTCAAGTGCATGTTGGCCTT	
	Inner Forward	CCTGATGATCGTAGGCATCG	
	Probe	TACCAGGAGGCAAATGGCACCC 5-FAM/3-BHQ	
	Inner Reverse	TGCATTTCAGGGCAAAGATG	
<i>Three Gene Mean</i>	POSTN	Outer Forward	GCAAACCACCTTACGGATCT
		Outer Reverse	TTATTCACAGGTGCCAGCAAAG
		Inner Forward	CGGATCTTGTGGCCCAATT
		Probe	CTTGGCATCTGCTCTGAGGCC 5-FAM/3-BHQ
		Inner Reverse	AGGTGCCAGCAAAGTGATTCTC
	CLCA1	Outer Forward	CCAGGCATTGCTAAGGTTGG
		Outer Reverse	ACTGGCCCTGAGAATTGGG
		Inner Forward	CCTTGACCCTGACTGTCACGT
		Probe	TGCGTCCAATGCTACCCTGCCTC 5-FAM/3-BHQ
		Inner Reverse	TTGTTTCGTTTTGGAAGTCACTGTAA
	SERPINB2	Outer Forward	CTGAAGTGTCCACCAAGCCA
		Outer Reverse	CAAAGTGTGGCCTCCATGT
		Inner Forward	GTGAATGAGGAGGGCACTGAA
		Probe	TAACACCTCCTGTGCCAGCGGCTG 5-FAM/3-BHQ
		Inner Reverse	CCATGTCCAGTTCCTCCTGTC

House-keeping Genes

RPL13A	Outer Forward	GGACCGTGCGAGGTATGCT	
	Outer Reverse	TTCAGACGCACGACCTTGAG	
	Inner Forward	TATGCTGCCCCACAAAACC	
	Probe	CAGAGCGCCTGGCCTCGCT	5-FAM/3-BHQ
	Inner Reverse	TGCCGTCAAACACCTTGAGA	
PPIA	Outer Forward	ATGAGAACTTCATCCTAAAGCATACG	
	Outer Reverse	TTGGCAGTGCAGATGAAAACT	
	Inner Forward	ACGGGCCTGGCATCTTGT	
	Probe	ATGGCAAATGCTGGACCCAACACA	5-FAM/3-BHQ
	Inner Reverse	GCAGATGAAAACTGGGAACCA	
EEF1A1	Outer Forward	TGCTAACATGCCTTGTTCAAG	
	Outer Reverse	TTGGACGAGTTGGTGGTAGGAT	
	Inner Forward	CCTTGTTCAAGGGATGGAA	
	Probe	CACTGGCATTGCCATCCTTACGGG	5-FAM/3-BHQ
	Inner Reverse	GCCTCAAGCAGCGTGGTT	